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Influence of seasonal differences on semen quality and subsequent embryo development of Belgian Blue bulls

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ABSTRACT

Belgian Blue bulls are more susceptible to high temperature and humidity index (THI) than most other cattle breeds. Here, we investigated whether high ambient temperature during summer affected semen quality and subsequent embryo development in Belgian Blue cattle. For this purpose, semen samples were collected from six healthy mature Belgian Blue bulls in March (Low THI group; THI between 30.6 and 56.4) and August 2016 (High THI group; maximum THI of 83.7 during meiotic and spermiogenic stages of spermatogenesis; 14–28 days prior to semen collection) respectively. Motility, morphology, acrosome integrity, chromatin condensation, viability, and reactive oxygen species production were assessed for frozen-thawed semen. Moreover, the efficiency of blastocyst production from the frozen-thawed semen samples of the two groups was determined *in vitro*. Blastocyst quality was determined by assessing inner cell mass ratio and apoptotic cell ratio. Fresh ejaculates showed a higher sperm concentration in low THI when compared to the high THI group ($P \leq 0.05$), whereas semen volume, subjective motility, and total sperm output were not affected ($P > 0.05$). In frozen-thawed semen, total and progressive motility, viability, and straight-line velocity were lower in high THI compared to the low THI group ($P < 0.05$), while H_2O_2 concentration, aberrant chromatin condensation, and abnormal spermatozoa were higher in the high THI group ($P < 0.05$). Blastocyst rates were significantly higher when low THI samples were used ($P < 0.05$). Moreover, the total cell number and trophectoderm cells were significantly higher ($P < 0.05$) in blastocysts derived from low THI samples, whereas the apoptotic cell ratio was significantly higher ($P < 0.01$) in blastocysts derived from high THI spermatozoa. In summary, our data show that elevated ambient temperature and humidity during summer can decrease the quality of frozen-thawed spermatozoa in Belgian Blue bulls and also affect subsequent embryo development.
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1. Introduction

The accumulation of greenhouse gases in the atmosphere is the most important cause of global warming. Global warming is not only restricted to the tropical, arid or Mediterranean zones, but has also become visible in regions in Europe with a traditionally moderate climate [1,2]. The temperature humidity index (THI), which was first developed by Thom [3], combines the effects of temperature and relative humidity to quantify the degree of heat stress on farm animals [4,5]. The THI thresholds for bull semen production in European temperate regions, where bulls of *Bos Taurus* breeds are kept [6] are much lower compared to those reported for tropical and subtropical conditions, where predominantly *Bos indicus* cattle are present [7,8].

High ambient temperature is inducing an increase in body temperature leading to lower production [9] and impaired reproduction results [10]. Ruminants present a relatively higher sensitivity to heat stress than other mammals because of their extra thermogenic activity due to the forage rumination and rumen fermentation [11]. Bulls exposed to summer heat stress show an

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increase of respiratory rate and rectal temperature [12], reduction in testis weight, alteration in testosterone production [13], reduction in sperm concentration, motility, plasma membrane integrity, and increase the possibility of mutations in sperm DNA as well as morphologically abnormal spermatozoa [14,15]. These can adversely affect bovine embryo development, leading to low conception and fertility rates [16,17]. In several mammalian species, elevated temperatures can decrease sperm quality by increasing morphological abnormalities in semen of mice [18], ram [19], stallions [20] and bulls [21] leading to poor fertility results either *in vitro* or *in vivo* [17,22]. During heat exposure, animals regulate their body temperature by a physiological adjustment which leads to increased generation of reactive oxygen species (ROS) [14,18], being the first pathway affecting the quality of spermatozoa [19,23]. The effect of heat stress on bull sperm quality has been studied by scrotal insulation [24,25]. Although these studies planned to simulate scrotal heat stress, but they do not accurately reflect alterations in sperm quality that might occur due to natural heat stress during summer because they don't simulate the normal testicular temperature regulation mechanisms [16].

Spermatogenesis in bulls lasts approximately 60 days, including three different stages: spermatocytogenesis, meiosis, and spermiogenesis. This process is followed by a period of epididymal maturation to produce functional spermatozoa [26]. Other researchers have demonstrated that pachytene and diplotene spermatocytes as well as early round spermatids are most sensitive to heat stress in bulls [2,27], in contrast to epididymal spermatozoa, which remained mostly unaffected [28]. Therefore, detrimental effects of high THI will only start from two weeks after heat exposure and remain present for at least eight weeks [14]. Consequently, the effect of environmental temperature on sperm quality may be missed if sperm quality evaluation is made at an inappropriate time [23].

Belgian Blue bulls are typically double-muscled and represent the main beef source in Belgium. Their extreme muscularity and small scrota, as well as their lower testicular thermoregulatory capability [24] makes this breed more susceptible to the heat stress than other breeds [29,30], especially in Belgian humid weather. Rahman et al. [24] showed that Belgian Blue bulls appear to be more vulnerable to heat stress than Holstein bulls. Moreover, Hoflack et al. [30] indicated that Belgian Blue bulls generally have lower sperm quality than Holstein bulls. To the best of our knowledge, there is a lack of information regarding the evaluation of the effect of high THI on Belgian Blue bull semen quality and subsequent *in vitro* and *in vivo* fertility. Therefore, the purpose of this study was to investigate the effect of high THI during summer on the sperm quality and subsequent *in vitro* embryo development of Belgian Blue bulls.

2. Materials and methods

2.1. Chemical and reagents

Phosphate-Buffered Saline [PBS, catalogue number (catnr) 20012019] and CellROX® (catnr C10429), basal medium eagle amino acids, minimal essential medium non-essential amino acids (100X), TCM-199 medium, kanamycin and gentamycin were purchased from Life Technologies Europe (Ghent, Belgium). Fetal calf serum (FCS) was obtained from Greiner Bio-one (Wemmel, Belgium). Chromomycin A_3 (CMA_3, catnr 7059-24-7), 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA, catnr 4091-99-0), propidium iodide (PI, catnr 25535-16-4) and all other chemicals not otherwise listed were obtained from Sigma-Aldrich (Diegem, Belgium). All media were filtered through a sterile filter (0.22 μm, Millipore Corporation, USA).

2.2. Animals and semen collection

Belgian Blue bulls ($n = 6$, age 2–7 years old) were maintained under uniform feeding and management conditions in each season. The bulls were housed in individual indoor box stalls and fed twice per day a TMR and had ad libitum access to water and forage in an adjacent paddock. Neither the temperature nor humidity was modified with the use of fans or air conditioning. Semen samples were collected from these six bulls in March and August 2016 via an artificial vagina (twice a week, at least five ejaculates for each bull in each season) in a Belgian breeding company (AWE, Ciney, Belgium,50°29 N, 5°11 E). The semen samples were by well-trained technicians through a standard protocol with a standard checklist. Semen collection also performed with an equal time interval.

The days of collection were selected to reflect the time lag between heat stress and an effect on sperm quality: the effect becomes visible 14–42 days after high THI [23,24]. Based on the meteorological data [31], three consecutive days were selected in July 2016, with maximum temperature and maximum relative humidity (RH) more than 30 °C and 85% respectively, and a THI oscillating between 63.5 and 83.7 (Figs. 1 and 2). This range includes mild and severe heat stress conditions for Belgian Blue bulls, taking into account that, the optimal environment for bull semen production in European temperate climate conditions is estimated to range between 15 and 18 °C and THI ranging from 50 to 60 for the entire spermatogenesis period [6]. Moreover, the semen samples of the low THI group were collected in March under a maximum temperature and RH of 8 °C and 84% respectively; and the THI values were oscillating between 30.6 and 56.4. The meteorological data were provided for Ciney city by the Accuweather web site [31] and THI indexes were calculated using the following equation where RH is relative humidity, T is temperature and THI is Temperature Humidity Index [32].

$$THI = (1.8 \times T + 32) - (0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)$$

2.3. Fresh semen parameters

Within 5 min of semen collection, fresh semen quality parameters were determined (five ejaculates/bull in each season, 30 samples for each treatment). The sperm concentration was measured using an Accucell photometer (IMV Technologies, L'Aigle, France). Moreover, individual motility of the spermatozoa was measured by phase-contrast microscopy (1:100 dilution in saline) and was expressed as the proportion of motile spermatozoa (percentage).

2.4. Sperm cryopreservation

Samples with sperm motility over 60% and concentrations higher than 0.3×10^9 spermatozoa/mL were further extended in Optidyl™ (Cryo-Vet, Québriac, France) extender and cooled to 4 °C for 4 h in a fridge (0.125 °C/min). Then, the extended semen samples were loaded into 0.25 mL straws (100×10^6 spermatozoa/straw) at 4 °C, and freezing was performed with a programmable freezing system (IMV, Technologies-Digitcool, L'Aigle, France). Briefly, the straws were cooled to −10 °C at −5 °C/min, from −10 °C to −140 °C at −40 °C/min, and were subsequently plunged into liquid nitrogen until further analysis.

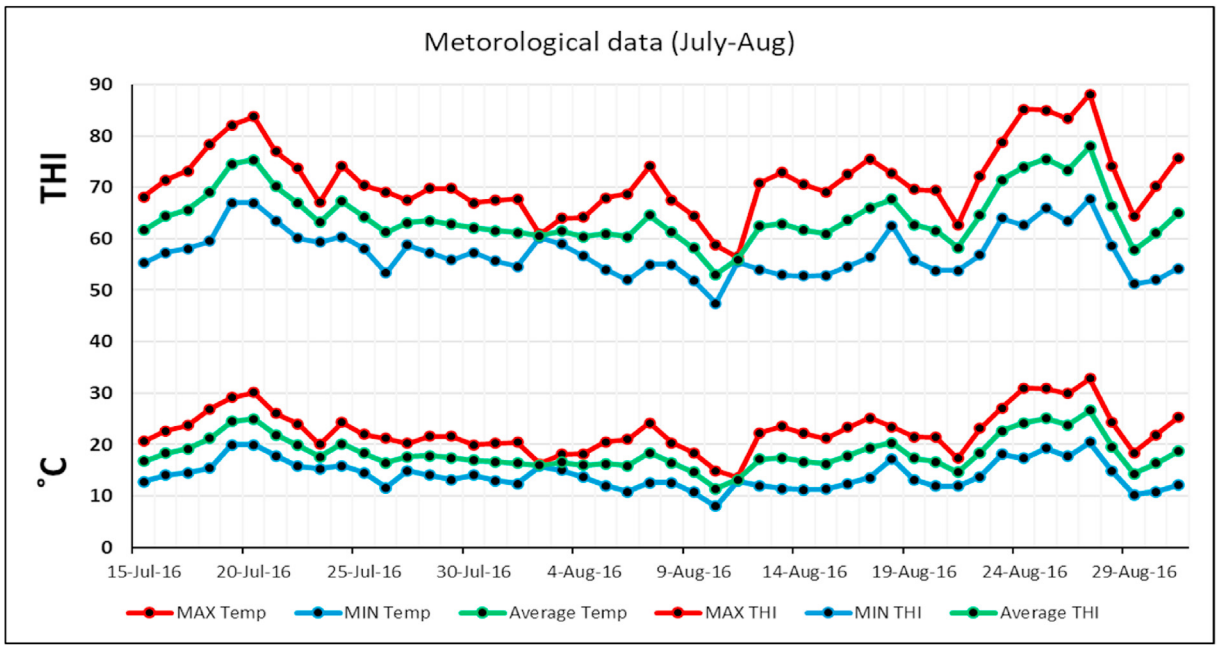

Fig. 1. Daily minimum and maximum temperature and humidity index in July and August 2016 (Ciney, Belgium).

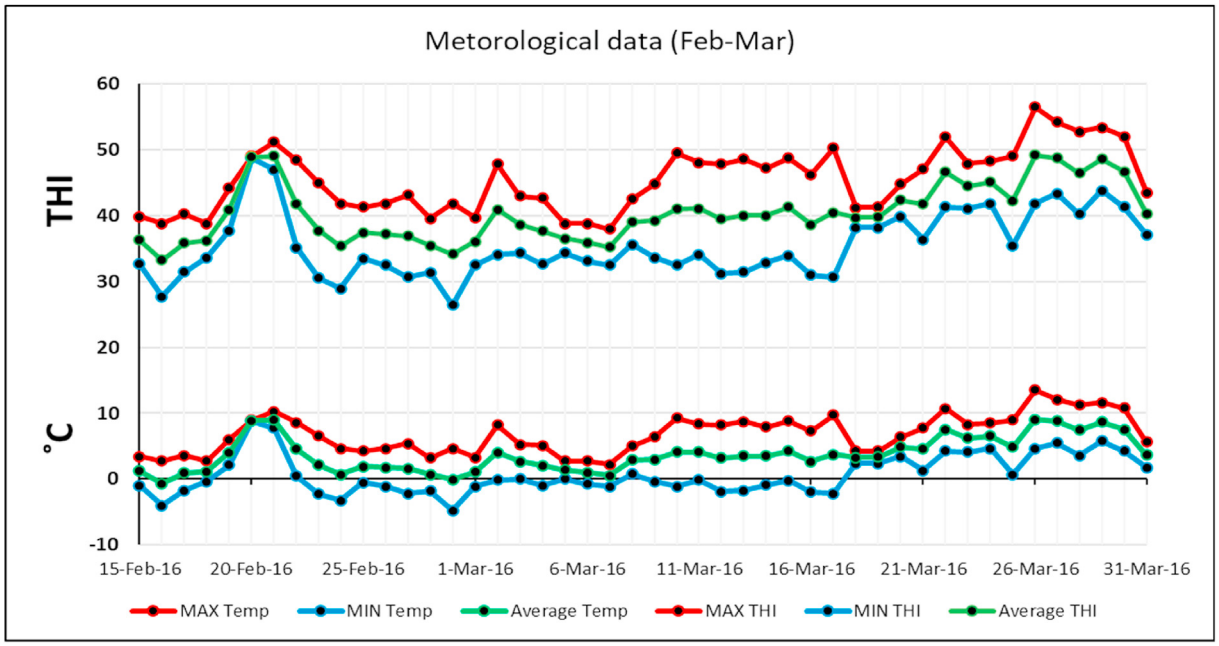

Fig. 2. Daily minimum and maximum temperature, and THI in februay and March 2016 (Ciney, Belgium).

2.5. Assessment of motility parameters and morphological abnormalities

Frozen sperm samples of all experimental bulls (five ejaculates/bull in each season) were thawed at 37 °C for 30 s in a water bath. Subsequently, the thawed samples were diluted with buffer (Easybuffer B, IMV Technologies, L'Aigle France) at 1:3 proportion and held for 5 min on warm plate. Then a droplet (3 μL) of the diluted sample was placed in a pre-warmed Leja chamber (37 °C; Leja counting chambers, depth 20 mm; Microptics, Barcelona, Spain) and automatically assessed for motility and morphology using Computer Assisted Sperm Analyzer (CASA) system (IVOS-II CASA system, Hamilton Thorne Inc, Beverly, USA). At least 1000 spermatozoa/sample in eight microscopic fields were analyzed at a magnification of 100X. The assessed parameters included total and progressive motility (%), average path velocity (VAP; μm/s), curvilinear velocity (VCL; μm/s), straight-line velocity (VSL; μm/s), straightness (STR, VSL/VAP), linearity (LIN, VSL/VCL), amplitude of lateral head deviation (ALH, μm), and beat cross frequency (BCF, Hz). Moreover, the assessed morphological abnormalities were percentage of spermatozoa with abnormalities, bent tails, coiled tails, distal and proximal protoplasmic droplets. The settings of the CASA software for evaluating the motility kinetics and morphological abnormalities are presented in Table 1.

Table 1
CASA software settings for bull semen.

Parameter*	Parameter function	Value
Frame rate (Hz)	Image capture	60
Number of frames captured	Image capture	30
Minimum contrast	Cell detection	20
Minimum cell size (pixels)	Cell detection	5
Straightness cut-off (%)	Progressive cell detection	80
Fast VAP cut-off (μm/s)	Progressive cell detection	50
Low VAP cut-off (μm/s)	Static cell detection	20
Static VAP cut-off (μm/s)	Static cell detection	5
Low VSL cut-off (μm/s)	Static cell detection	2

*CASA, computer assisted sperm analyzer; VAP, average path velocity (μm/s); VSL, straight line velocity (μm/s); STR, straightness (VSL/VAP, %).

2.6. Assessment of acrosome integrity

The acrosome integrity of spermatozoa was assessed using peanut agglutinin conjugated to fluorescein isothiocyanate (PNA-FITC; Sigma-Aldrich, St. Louis, MO). Briefly, thawed samples (three ejaculates/bull in each season) were washed twice in Ca^{2+} Mg^{2+} free Phosphate Buffer Saline (PBS, proportion 1:5) at 600 g for 10 min. Next, spermatozoa were stained with 1 μg/mL PNA-FITC for 15 min at room temperature (RT). After two extra washes with PBS, the stained spermatozoa were observed using a Leica DMR microscope equipped with a mercury lamp and appropriate filters, at a magnification of 400X. To discriminate the spermatozoa with intact acrosome from acrosome-damaged spermatozoa, at least 200 spermatozoa were counted and the relative percentage of intact acrosome spermatozoa was calculated. Spermatozoa without PNA-FITC-labeled acrosome regions were considered as intact, whereas spermatozoa with fluorescence over the acrosomal region were considered to have reacted acrosomes [33,34].

2.7. Assessment of sperm chromatin condensation

Sperm chromatin condensation was assessed using CMA_3. Briefly, thawed samples (three ejaculates/bull in each season) were washed twice in PBS at 600 g for 10 min, and subsequently fixed in Carnoy's solution (3:1 methanol and acetic acid) at 4 °C for 5 min. The sperm pellets were then resuspended in three droplets of PBS (30 μL) and stained with CMA_3 stock solution (100 μL of 0.25 mg/mL CMA_3 in McIlveen solution; 7 mL citric acid 0.1 M + 32.9 mL $Na_2HPO_4 \cdot 7H_2O$ 0.2 M, pH 7, containing 10 mM $MgCl_2$) for 20 min at RT in the dark [35]. Samples were labeled with Hoechst 33342 (5 mg/mL) for 5 min to determine the total number of spermatozoa. Then, the sperm samples were washed to remove the excess of CMA_3, air-dried and mounted on a slide with 1,4-diazabicy-clo (2.2.2) octane solution (DABCO) on a glass slide. At least 200 spermatozoa per slide were counted at 200X magnification using an epifluorescent (Olympus I×80, Olympus Corporation) microscope. Bright yellowish-green spermatozoa showed aberrant chromatin condensation while dull green labeling showed normal chromatin condensation [24].

2.8. Flow cytometry analysis of frozen-thawed semen

2.8.1. Sample preparation

Thawed samples (four ejaculates/bull in each season) were washed twice in PBS, resuspended to a final concentration of 2.5×10^6 spermatozoa/mL and subsequently divided in 200 μL aliquots for further flow cytometric assessment.

2.8.2. Analysis

Flow cytometric analysis was performed with a CytoFLEX flow cytometer (Beckman Coulter Inc., Atlanta, Georgia, USA) equipped with three lasers (405 nm, 488 nm, 638 nm) and calibrated daily with CytoFLEX Daily Q fluorescent microbeads. Cell fluorescence of the samples was excited at 488 nm. The fluorescence intensity of the PI and FITC (CellROX and DCFH-DA) channels was measured to analyze the percentage of positive cells for cell death (PI) and ROS production (CellROX and DCFH-DA) in low THI versus high THI semen samples. The emission of the PI and FITC channels were 585/42 nm and 525/40 nm, respectively. All data were corrected for autofluorescence and single-stained controls were used to set compensation values for PI and FITC channels. The single sperm population was gated on FSC-H versus FSC-A dot plot to exclude aggregates/interconnected spermatozoa, and on FS/SS dot plot to exclude debris [35]. A total of 10,000 events (spermatozoa) were recorded and analyzed at flow rate of 30 μL/min. For quantification of the data, the data were processed using CytExpert v2.0.0.153 software (Beckman Coulter, Inc., California, USA). Dot plots of PI vs FITC were created and divided into 4 quadrants to analyze the different subpopulations (Fig. 3).

2.8.3. Assessment of oxidative status

After sample preparation, 200 μL of suspended sperm samples (0.5×10^6 spermatozoa) were aliquoted for each parameter. For oxidative stress evaluation, the hydrogen peroxide (H_2O_2) concentration was assessed by the conversion of 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to DCF, while the superoxide onion (O_2^-) free radicals' level was assessed using CellROX green probes [35,36]. Briefly, one sperm sample (200 μL) was incubated with 5 μM CellROX while a second sample was incubated with 100 μM DCFH-DA. Both stained sperm samples were incubated for 30 min at 37 °C in the dark. The sperm suspension was washed twice in PBS at 270 g for 5 min, and 1.5 μM of PI was added to assess sperm viability. Furthermore, one group of unstained samples was added as autofluorescence group. Finally, the samples were loaded in a 96 well plate and analyzed using a flow cytometer. To interpret the ROS generation data, a dot plot of two fluorescence channels PE (PI) and FITC (DCF or CellROX) was distributed in different quadrants (Fig. 3). The first quadrant (Lower Left, LL) represented PI^-/DCF^- or PI^-/$CellROX^-$ which is the unstained viable sperm subpopulation without ROS production. The second quadrant (Upper left, UL) corresponds to PI^+/DCF^- or $PI^+/CellROX^-$, dead spermatozoa without ROS production. The third quadrant (Lower right, LR) corresponds to the PI^-/DCF^+ or $PI^-/CellROX^+$, alive spermatozoa producing ROS. The fourth quadrant (Upper right, UR) corresponds to PI^+/DCF^+ or $PI^+/CellROX^+$, the population of dead spermatozoa that produced ROS. For data analysis, the 3rd population (PI^-/DCF^+ and $PI^-/CellRox^+$) was selected which represents viable spermatozoa producing ROS.

2.8.4. Assessment of sperm viability

Live/dead spermatozoa were assessed using PI. Briefly, 200 μL resuspended sperm samples (2.5×10^6 spermatozoa) were stained with 1.5 μM PI and incubated for 5 min at 37 °C in the dark. Then the samples were washed twice, resuspended in PBS and analyzed by flow cytometry. To prepare the viability dataset, the histograms of PI fluorescence were analyzed and divided in two subpopulations, i.e. PI^- and PI^+, which represent the living and dead spermatozoa, respectively.

2.9. In vitro embryo production

Bovine embryos were produced by routine *in vitro* methods as described before [37]. Briefly bovine ovaries were collected from a local slaughterhouse and processed within 2 h after collection. Upon arrival, the excised ovaries were rinsed three times in a warm

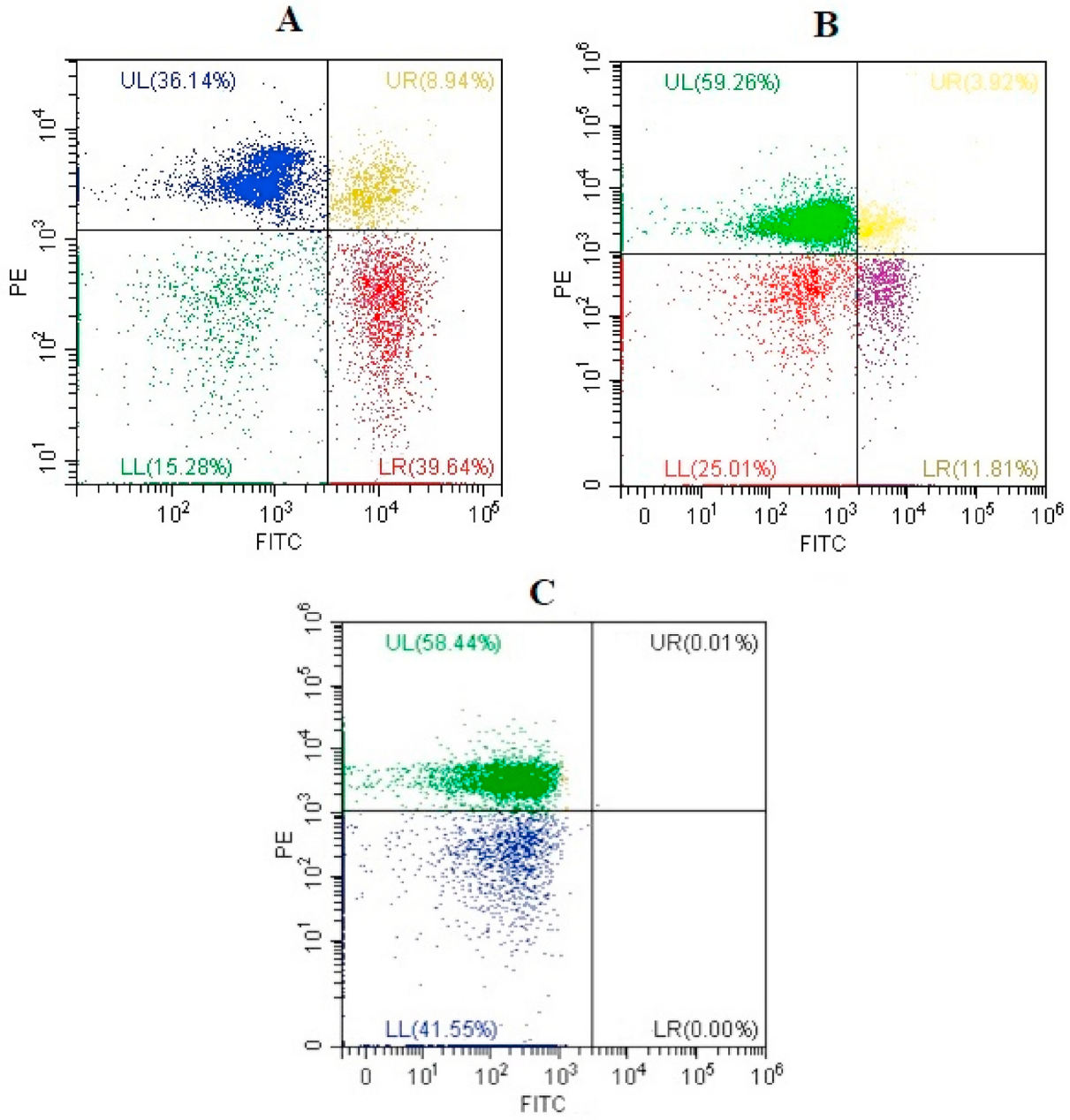

Fig. 3. Representative examples of flow cytometry analyses assessing the physiology of thawed bull spermatozoa. (A) Cytogram of bull spermatozoa O*- free radicals production evaluated with CellROX green probe, (B) Scatter plot of H_2O_2 production evaluated via DCFH-DA probe, (C) cytogram of thawed spermatozoa viability assessed by PI. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

physiological saline (0.9% NaCl) supplemented with 25 mg/mL kanamycin. Then immature cumulus-oocyte complexes (COCs) were obtained from 2 to 8 mm antral follicles using an 18-gauge needle attached to a 10 mL syringe. Oocytes surrounded with at least three layers of non-expanded cumulus cells and with uniform ooplasm, were cultured in groups of 60 in 500 mL of bicarbonate-buffered TCM199 medium (supplemented with 50 mg/mL gentamycin and 20 ng/mL of epidermal growth factor) in 5% CO_2 in the air for 22 h at 38.5 °C.

Frozen spermatozoa from three bulls collected in two different seasons (March vs August) were thawed and separated using 45/90% Percoll gradient (GE Healthcare Biosciences, Uppsala, Sweden). Afterward, sperm pellet was washed in Tyrode's albumin–pyruvate–lactate (TALP) medium, containing bicarbonate-buffered Tyrode solution and adjusted to a final concentration of 2×10^6 spermatozoa/mL using IVF–TALP medium enriched with 6 mg/mL BSA and 25 mg/mL heparin.

Oocytes with expanded cumulus cells were washed in 500 μL of IVF-TALP medium after 22 h maturation, and co-incubated with washed spermatozoa (in a well containing 500 μL of IVF-TALP medium with 25 matured oocytes and at least 0.5×10^6 spermatozoa/well) for 21 h. Afterward, presumed zygotes were vortexed to remove excess spermatozoa and zona-attached cumulus cells and cultured in groups of 25 in 50 μL droplets synthetic oviductal fluid enriched with non-essential and essential amino acids (SOFaa), and ITS (5 μg/mL insulin; 5 μg/mL transferrin; 5 ng/mL selenium).

Medium droplets were covered with mineral oil and incubated at 38.5 °C in 5% CO_2, 5% O_2 and 90% N. This experiment was repeated four times.

Embryonic development was evaluated by assessing the cleavage rate (the percentage of cleaved embryos out of presumed zygotes) at 45 h post insemination (hpi), blastocyst rates [the percentage of blastocysts out of presumed zygotes on day 7 and 8 post insemination (dpi)] and hatching rates (the percentage of hatching/hatched blastocysts out of the total number of blastocysts at eight dpi) [38].

2.10. Blastocyst quality assessment

Blastocyst quality was assessed by differential staining [37]. Briefly, day 8 blastocysts (Totally 98 blastocysts) were fixed for 20 min in 4% paraformaldehyde (*w/v*) and stored at 4 °C. At the time of staining, blastocysts were permeabilized with 0.5% Triton X-100 + 0.05% Tween for 1 h at RT. For denaturation of the DNA, the blastocysts firstly were exposed to 2 N HCl for 20 min, and then immerged in 100 mM Tris-HCl (pH = 8.5) for 10 min at RT. Afterward, the embryos were washed in PBS and incubated in blocking solution (containing 10% goat serum and 0.5% BSA prepared in PBS) at 4 °C overnight. Next, embryos were washed in PBS and incubated in blocking solution supplemented with primary CDX_2 antibody (Biogenex, San Ramon, USA) at 4 °C overnight. Then the blastocysts were washed and incubated in blocking solution containing rabbit active caspase-3 antibody (0.768 mg/mL, Cell Signaling Technology, Leiden, the Netherlands) overnight. After washing, they were incubated in blocking solution consisting of goat anti-mouse Texas Red secondary antibody (20 mg/mL, Molecular Probes, Merelbeke, Belgium) for 1 h and exposed to goat anti-rabbit FITC antibody (10 mg/mL in blocking solution, Molecular Probes) for another 1 h at RT. This staining protocol allows instantaneous evaluation of the total cell number (TCN), trophectoderm cells number (TE) the proportion of inner cell mass (ICM) ratio (ICM/TCN) and the apoptotic cell ratio (ACR), the percentage of apoptotic cells relative to the TCN.

2.11. Statistical analysis

The dataset was analyzed using the GLM procedure of SAS software [SAS Institute Version 9.1 (TS M0), 2002, Cary, NC, USA]. Normal distribution of the dataset was checked using UNIVARIATE procedure, Shapiro-Wilk test and Arcsine square root transformation were used when required [39]. For all of the parameters, treatments (high THI vs low THI) were considered as fixed factor in the model equation. Furthermore, for controlling the individual effect of the bulls in the statistical analysis, their effect was considered as a random factor in the model equation. The results were expressed as Means ± SE and Duncan test were applied to determine the significant differences. The differences with *P* values < 0.05 were considered as significant.

3. Results

3.1. Fresh semen quality

Season had no effect on semen volume, total sperm output (TSO) and subjective motility ($P > 0.05$), whereas sperm concentration was negatively affected in high THI group, as the high THI samples showed a lower ($P < 0.05$) sperm concentration compared to low THI group. Moreover, the bull effect on fresh semen parameters was significant. An interactive effect between bull and season was statistically significant for both semen volume and sperm concentration but not significant for subjective motility (Table 2).

3.2. Post-thawed semen quality parameters and subsequent embryonic development

3.2.1. Sperm motility characteristics

The impact of high THI on semen motility after thawing is presented in Table 3. The frozen-thawed low THI spermatozoa had higher TM, PM, and VSL compared to high THI group ($P < 0.05$), but VCL, VAP and STR did not significantly differ between both groups. Furthermore, interactive effect between bull and season was not statistically significant for all parameters.

3.2.2. Sperm morphological abnormalities

The effect of high THI on sperm morphology after freezing and thawing is shown in Table 4. The results show that high THI spermatozoa had higher percentage of abnormal spermatozoa when compared to low THI ($P < 0.05$). Moreover, the percentage of spermatozoa showing a bent tail and a distal protoplasmic droplet was also significantly higher in the high THI samples, but all other morphological parameters did not differ between the two groups ($P > 0.05$).

3.2.3. Sperm acrosome status and chromatin condensation

The effect of high THI on sperm chromatin condensation and acrosome integrity after thawing are shown in Table 5. The percentage of spermatozoa with aberrant chromatin condensation was lower in low THI group ($P < 0.01$), but the percentage of spermatozoa with damaged acrosomes did not differ between both experimental groups ($P > 0.05$). The effect of bull ($P = 0.97$) and the interaction between bull and treatment on acrosome status was also not statistically significant (see Table 5).

3.2.4. Sperm viability and oxidative status

Effect of high THI on sperm viability, O_2^- and H_2O_2 production after freezing and thawing is presented in Table 5. The elevated environmental temperature during summer was associated with increased sperm H_2O_2 production ($P < 0.05$) which resulted in a decrease in sperm viability ($P < 0.05$) but the production of O_2^- free radicals did not statistically differ between the two groups. Moreover, the interactive effects between bull and treatment on viability, O_2^- and H_2O_2 production were not statistically significant (Table 5).

Table 2
Effect of high THI on Belgian Blue bull's fresh semen parameters (Mean ± SE).

Parameters	Treatment		*P*-Value		
	Low THI	High THI	Treatment	Bull	Treatment × bull
Subjective Motility (%)	70.33 ± 5.17	69.67 ± 6.05	0.52	<0.01	0.45
Semen Volume (mL)	8.36 ± 2.23	8.52 ± 2.29	0.49	<0.01	0.01
Concentration (10^9/mL)	1.57[a] ± 0.04	1.43[b] ± 0.05	0.04	<0.01	0.01
TSO (× 10^9)	12.47 ± 2.62	11.70 ± 2.51	0.19	<0.01	0.42

SE, standard error; THI, temperature humidity index; TSO, total sperm output (sperm concentration × semen volume).
[a] and [b]: Different superscripts show significant differences between treatments ($p \leq 0.05$).

Table 3
Effect of high THI on frozen-thawed sperm motility parameters of Belgian Blue bulls (Mean ± SE).

Parameters	Treatment		*P*-Value		
	Low THI	High THI	Treatment	Bull	Treatment × bull
TM (%)	52.36[a] ± 1.31	42.23[b] ± 1.26	<0.01	<0.01	0.23
PM (%)	32.39[a] ± 0.77	24.41[b] ± 1.10	<0.01	<0.01	0.90
VCL (μm/s)	166.69 ± 3.43	160.92 ± 3.50	0.26	0.06	0.85
VSL (μm/s)	76.79[a] ± 1.60	71.47[b] ± 1.91	0.03	0.13	0.93
VAP (μm/s)	91.45 ± 1.74	86.74 ± 1.93	0.07	0.15	0.95
STR (%)	46.65 ± 0.51	45.43 ± 0.72	0.05	0.01	0.35
LIN (%)	82.08 ± 0.39	80.83 ± 0.74	0.06	0.02	0.32
ALH	7.11 ± 0.14	7.04 ± 0.15	0.72	<0.01	0.54
BCF	31.20[a] ± 0.38	29.72[b] ± 0.45	0.02	0.05	0.49

ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, Linearity; LS, least square; PM, progressive motility; SE, standard error; STR, straightness; THI, temperature humidity index; TM, total motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.
[a] and[b]: Different superscripts show significant differences between treatments ($p \leq 0.05$).

Table 4
Effect of high THI on morphological abnormalities in frozen-thawed spermatozoa of Belgian Blue bulls (Means ± SE).

Parameters	Treatment		*P*-Value		
	Low THI	High THI	Treatment	Bull	Treatment × bull
Total abnormality (%)	7.44[b] ± 0.74	9.16[a] ± 0.48	0.001	<0.01	0.57
Bent tail (%)	1.99[b] ± 0.14	2.80[a] ± 0.12	<0.01	0.04	0.10
Coiled tail (%)	0.28 ± 0.03	0.30 ± 0.03	0.62	0.60	0.47
Distal droplet (%)	2.70[b] ± 0.19	3.26[a] ± 0.18	<0.01	<0.01	0.15
Proximal droplet (%)	3.12 ± 0.53	2.74 ± 0.33	0.11	<0.01	0.95

LS, least square; SE, standard error; THI, temperature humidity index.
[a] and[b]: Different superscripts show significant differences between treatments ($p \leq 0.05$).

Table 5
Effect of high THI on acrosome status and protamine deficiency, viability, O_2^- and H_2O_2 production in frozen-thawed spermatozoa of Belgian Blue bulls (Means ± SE).

Parameters (%)	Treatment		*P*-Value		
	Low THI	High THI	Treatment	Bull	Treatment × bull
Aberrant chromatin condensation	2.27[b] ± 0.26	3.86[a] ± 0.21	<0.01	0.63	0.97
Damaged acrosome	27.89 ± 0.84	29.80 ± 0.79	0.11	0.96	0.14
Viability (PI^-)	54.32[a] ± 1.27	46.30[b] ± 1.24	<0.01	0.01	0.62
O_2^- production ($PI^-/CellROX^+$)	17.06 ± 2.52	21.16 ± 2.97	0.19	<0.01	0.96
H_2O_2 production (PI^-/DCF^+)	17.75[b] ± 1.85	26.67[a] ± 2.41	<0.01	0.11	0.98

DCF, dichlorodihydrofluorescein; H2O2, hydrogen peroxide; O2-, superoxide onion; PI, propidium iodide; SE, standard error; THI, temperature humidity index.
[a] and[b]: Different superscripts show significant differences between treatments ($p \leq 0.05$).

3.2.5. Embryonic development

Cleavage and blastocyst rates at Day 7 and 8 were higher in the group derived from low THI spermatozoa ($P < 0.05$). However, there was no significant difference in hatched/hatching rate between treatments (Table 6). The TCN and TE were higher in blastocysts derived from high THI samples ($P < 0.05$). The ACR was lower ($P < 0.001$) in blastocysts derived from low THI samples (Table 7). The results indicated that the day 8 blastocysts rate of all individual bulls was significantly higher when the semen samples were collected during low THI period (Table 8).

4. Discussion

The present study was conducted to verify whether seasonal differences had an effect on the quality of fresh and frozen-thawed semen of Belgian Blue bulls, and which parameters were most affected. The Seasonal factors including thermal condition, forage quality, atmospheric pressure and visible light length and quality are the most important factors affecting the reproductive parameters as well as sperm quality in cattle breeding [40]. Among these factors heat stress combined with higher relative humidity, is the major source of sperm quality reduction during spermatogenesis [4,5].

Here we focused on the effect of high THI on Belgian Blue bull spermatozoa. For this purpose, we selected three days with higher THI value in August, and the samples were collected about 30 days afterward to reflect the impact of high THI on bull spermatogenesis. The collected sperm samples assumed to be in the spermiogenesis period in the selected days [16,24]. Moreover, we investigated whether exposure of bulls to heat stress during spermatogenesis period would affect their ability to generate high quality blastocysts *in vitro*.

The results of this study indicated that TM, PM, VSL, VAP and STR in frozen-thawed spermatozoa of high THI group were significantly decreased compared to low THI group. Several hypotheses can explain these seasonal effects on sperm motility parameters after thawing. Mitochondrial damages in sperm caused by high THI could be a possible reason for the reduced motility, as sperm motility is strongly dependent on the ATP produced by mitochondrial oxidative phosphorylation [15]. Increased testicular temperature during summer months can affect sperm quality of bulls [11,24] and rams [19] leading to lower conception rates. In agreement with the results of the current study, Malama et al. [41] reported that frozen-thawed spermatozoa collected in summer from Holstein bulls showed lower motility, acrosome integrity and mitochondrial function after freeze-thawing. Elevated

Table 6
Effect of high THI on embryo development of frozen-thawed spermatozoa (Means ± SE).

Parameters	Treatment		P-Value		
	Low THI	High THI	Treatment	Bull	Treatment × bull
Cleavage rate (%)	70.40[a] ± 0.74	62.79[b] ± 0.48	0.001	<0.01	0.57
Blastocyst rate (7 dpi, %)	25.40[a] ± 0.14	15.42[b] ± 0.12	<0.01	0.04	0.10
Blastocyst rate (8 dpi, %)	40.20 ± 0.03	23.31 ± 0.03	0.62	0.60	0.47
Hatched/hatching rate (%)	25.61[a] ± 0.19	22.83[b] ± 0.18	<0.01	<0.01	0.15

SE, standard error, THI, temperature humidity index.
[a] and[b] superscripts indicate significant difference (P ≤ 0.05) between sperm concentration and within the same bull.

Table 7
Total cell number, ICM cell number and rate (%), and apoptotic cell number and rate (%) of differentially stained day-8 blastocysts derived from low and high THI semen groups (Mean ± SE).

Parameter	Treatment		P-Value		
	Low THI	High THI	Treat	Bull	Treat × Bull
Blastocyst (n)	51	47	–	–	–
TCN (n)	119.03 ± 3.67[a]	100.63 ± 4.08[b]	<0.01	0.04	0.26
TE (n)	79.02 ± 3.16[a]	58.59 ± 3.15[b]	<0.01	<0.01	0.54
AC (n)	2.15 ± 0.47[b]	5.21 ± 0.52 [a]	<0.01	0.41	0.01
ICM (n)	40.02 ± 2.06	42.04 ± 2.41	0.6461	0.58	0.05
ICM/TCN (%)	34.18 ± 1.51[b]	42.38 ± 1.70[a]	<0.01	0.08	0.01
AC/TCN (%)	1.78 ± 0.39[b]	5.63 ± 0.65[a]	<0.01	0.03	<0.01

AC, Apoptotic cells; ICM, Inner cell mass; SE, standard error; TE, Trophectoderm cells; TCN, Total cell number; THI, Temperature humidity index.
[a] and[b]: Different superscripts show significant differences between treatments (p ≤ 0.05).

environmental temperature has been reported to decrease sperm motility, and increased the morphologically abnormal spermatozoa in ram [42] and bulls [11]. Moreover, Llamas Luceño et al. [17] showed that higher THI reduced sperm viability in Holstein bulls and led to a decreased subsequent blastocyst development. In comparison to other breeds, Belgian Blue bulls display impaired reproductive capacity, which seem to be associated with their double-muscled properties. They typically have a small scrotum with the absence of a distinct scrotal neck [30]. This anatomical peculiarity possibly increases their susceptibility to heat stress leading to lower fertility rate [20]. Moreover, Belgian Blue cattle belongs to the *Bos Taurus* breed, which is more sensitive to heat stress than *Bos indicus* subspecies and their crossbreds [43].

In the present study, the H_2O_2 content was increased in semen samples collected and frozen in summer. Furthermore, the elevated environmental temperature during summer was associated with sperm O-* production, albeit not significantly. Hence, the decreased sperm quality and subsequent embryo development may have been due to the increased production of ROS, causing lower viability, higher morphological abnormalities and higher aberrant chromatin condensation in the spermatozoa from high THI group. In general, increased testicular temperature leads to increased metabolism in the testes and consequently to a higher need for oxygen. Therefore, testes rely on increasing their blood flow to overcome heat stress-induced deleterious effects [44]. However, if the testicular blood flow is not able to meet this increased requirement, the testes became more hypoxic in response to the heat stress. Hypoxia can increase the ROS production by affecting the sperm mitochondrial electron transport chain [45]. In a recent review, Boni [10] stated that the deleterious effects of heat stress on reproductive functions in mammals are caused by increased ROS concentration in cells and tissues mitochondria according to a hypoxic condition. In addition, it is hypothesized that the ROS generated after heat stress may act as a cell death signal leading to increased spermatogenic cell death and their overproduction is associated with sperm membrane lipid peroxidation [46,47]. The results of our study are in accordance with those of Hamilton et al. [19] who observed that the testicular insulation decreased motility and increased the percentage of damaged plasma membrane, reacted acrosomes and spermatozoa with high mitochondrial membrane potential. They concluded that this alteration may be due to higher ROS levels in ejaculated semen.

The present study showed that the percentage of total abnormal morphological spermatozoa and the percentage of spermatozoa with bent tail and distal cytoplasmic droplet were higher in high THI group when compared to lower THI. Additionally, aberrant chromatin condensation was also increased in sperm samples collected in August. Lower chromatin compaction is an important indicator for protamine deficiency [24] which is closely associated with a higher DNA fragmentation index [48]. Likewise, Nuria-Llamas et al. [17] found that high THI during summer negatively altered head features and chromatin condensation of Holsteins bull spermatozoa. Similarly, an increase of morphologically abnormal, membrane damaged and DNA fragmented spermatozoa was observed 21 days after testicular insulation in rams [49]. Moreover, Rahman et al. [24] observed increased head abnormalities in bull spermatozoa two to six weeks after scrotal insulation, when the germ cells were presumably at the stages of spermiogenesis and meiosis. Increased morphological sperm abnormalities, especially head abnormalities after high THI exposure are assumed to be related to defective chromatin condensation during the acrosome and Golgi phase of spermiogenesis as spermatozoa are known to be vulnerable to heat stress when these cells are not completely protaminated [50]. Our findings are in accordance with the results of Barth and Waldner [51] who examined the prevalence and

Table 8
Total blastocyst development rates at day 7 and 8 after fertilization of embryos derived from low and high THI semen samples by individual bulls (Mean ± SE).

bulls	Oocytes		Cleavage rate		Day 7 blastocyst rate		Day 8 blastocyst rate	
	Low THI	High THI	Low THI	High THI	Low THI	High THI	Low THI	High THI
1	169	181	70.50[a] ± 1.68	63.04[b] ± 3.10	27.01 ± 3.17	18.51 ± 3.94	42.09 ± 2.52[a]	26.03 ± 4.42[b]
2	196	185	71.45 ± 1.21	66.11 ± 1.73	23.11 ± 3.87	15.47 ± 1.98	39.32 ± 1.87[a]	29.51 ± 4.06[b]
3	191	188	69.26[a] ± 3.00	59.22[b] ± 2.01	26.10 ± 3.10[a]	12.24 ± 0.89[b]	39.20 ± 1.71[a]	14.40 ± 1.93[b]
Average	–	–	70.40[a] ± 0.74	62.79[b] ± 0.48	25.40 ± 1.84[a]	15.42 ± 1.56[b]	40.20 ± 1.15[a]	23.31 ± 2.80[b]

SE, standard error; THI, temperature humidity index.
[a] and[b]: Different superscripts show significant differences between treatments (p ≤ 0.05).

importance of factors affecting semen quality and breeding soundness classification in beef bulls in Canada. They indicated that the percentage of different bull sperm with head defects was slightly lower in March and April than in June. Although the photoperiod, cold stress, and reduced feed quality may interact to reduce semen quality in the winter months, but in contrast with the results of Barth and Waldner [51] our results showed that the higher THI during summer months can decrease Belgian Blue bull semen quality.

The results of our study indicated that exposure of the bulls to high THI during summer can also negatively affect subsequent *in vitro* embryo development. The sperm quality alteration during spermatogenesis can affect quality of embryos as well as blastocyst development rates [52]. Heat stress as a consequence of high THI can alter the integrity of paternal DNA by protamine alteration [36], and may lead to disrupted expression of key developmental genes involved in formation of the blastocyst [53]. Similarly, in a previous study from our group, it has been shown that the high THI during summer negatively affected the viability of spermatozoa in Holstein bulls and led to a decrease in blastocyst development and delayed hatching [17].

5. Conclusion

In conclusion, exposure of Belgian Blue bulls to elevated environmental temperature negatively affected sperm quality. We observed an increased percentage of morphologically abnormal spermatozoa and increased ROS generation after thawing. Furthermore, there was a decline in sperm chromatin condensation, motility, viability, as well as augmented H_2O_2 generation. Spermatozoa collected after exposure to high THI, generated lower cleavage and blastocyst rates after *in vitro* fertilization. Further studies are needed to investigate the impact of heat stress as a consequence of high THI on the fertility of these semen samples after insemination in farm.

Credit author statement

Afshin Seifi-Jamadi: Conceptualization, Methodology, Investigation, Formal analysis, Writing- Original draft. **Núria Llamas Luceño:** conceptualization and Investigation. **Bart Leemans, Kristel Demeyere, Evelyne Meyer, Emilie Henrotte** and **Catherine Latour:** investigation. **Mahdi Zhandi, Hamid Kohram** and **Ann Van Soom**: supervision, Conceptualization, Methodology. All authors read the draft and revised the manuscript before the submission for publication.

In the memory of **Dr. Hamid Kohram** who has passed away because of COVID-19 virus pandemic. God bless him and rest in peace.

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